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Maintenance of Gammaherpesvirus Latency Requires Viral Cyclin in the Absence of B Lymphocytes

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Gammaherpesviruses establish a life-long chronic infection that is tightly controlled by the host immune response. We previously demonstrated that viruses lacking the gammaherpesvirus 68 (γHV68) viral cyclin (v-cyclin) exhibited a severe defect in reactivation from latency and persistent replication. In this analysis of chronic infection, we demonstrate that the v-cyclin is required for γHV68-associated mortality in B-cell-deficient mice. Furthermore, we identify the v-cyclin as the first gene product required for maintenance of gammaherpesvirus latency in vivo in the absence of B lymphocytes. While the v-cyclin was necessary for maintenance of latency in the absence of B cells, maintenance of v-cyclin-deficient viruses was equivalent to that of wild-type γHV68 in the presence of B cells. These results support a model in which maintenance of chronic γHV68 infection requires v-cyclin-dependent reactivation and reseeding of non-B-cell latency reservoirs in the absence of B cells and raise the possibility that B cells represent a long-lived latency reservoir maintained independently of reactivation. These results highlight distinct mechanisms for the maintenance of chronic infection in immunocompetent and B-cell-deficient mice and suggest that the different latency reservoirs have distinct gene requirements for the maintenance of latency.

The gammaherpesviruses are large, double-stranded DNA viruses that include the human pathogens Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV; also called human herpesvirus 8), as well as murine gammaherpesvirus 68 (γHV68), a closely related gammaherpesvirus that serves as a small-animal model. These viruses establish a lifelong infection in the host and encode many genes that manipulate the host cell machinery. A hallmark of chronic gammaherpesvirus infection is the ability of these viruses to establish a quiescent infection in host lymphocytes termed latent infection, which is characterized by maintenance of the viral genome as an episome, limited gene expression, and the absence of virus replication (25). Upon appropriate stimulation, latently infected cells can reactivate from latency, resulting in virus replication and production of infectious virus. During chronic infection, there may also be production of infectious virus at sites of persistent infection, allowing transmission to new hosts. EBV and KSHV persistently replicate at mucosal sites, including the oropharynx and genital tract (1, 25). γHV68 has been demonstrated to persistently replicate in the lungs, aorta, and peritoneal cells of immunocompromised mice (7, 16, 27, 30). Evidence of long-term T-cell stimulation in infected normal mice further supports the existence of ongoing persistent replication during chronic gammaherpesvirus infection (2, 3).

While gammaherpesvirus infection of immunocompetent individuals is typically controlled with few pathogenic outcomes, immune suppression can result in impaired control of chronic infection and lead to disease. Immune suppression and chronic infection with both EBV and KSHV results not only in well-recognized classical monoclonal lymphomas (8) but also in malignancies of mixed cell types, with inflammatory infiltrates and detectable reactivation within some cells including nasal pharyngeal carcinoma and Kaposi's sarcoma (1, 25). In addition, KSHV (24) and γHV68 (6, 31) have been associated with pneumonia during chronic infection of immunocompromised hosts. Chronic γHV68 infection of immunocompromised mice has also been demonstrated to result in large-vessel arteritis (42) and splenic fibrosis (12).

To characterize basic aspects of gammaherpesvirus pathogenesis, γHV68 infection of mice has been developed as a tractable small-animal model (26, 27). γHV68 is a gammaherpesvirus of rodents that can infect both inbred and outbred strains of mice and is genetically related to EBV and KSHV on the basis of shared genomic organization, conserved genes, and associated pathologies (39). Several of the gammaherpesviruses, including γHV68, KSHV, and the primate viruses herpesvirus saimiri and rhesus rhadinovirus, encode a viral cyclin that is homologous to host D-type cyclins (39). Previously, we demonstrated that transgenic expression of the γHV68-encoded cyclin homolog (v-cyclin) transforms primary cells (37). During virus infection of the host, the v-cyclin is essential for efficient reactivation from latency when cultured ex vivo. Cells latently infected with v-cyclin-deficient viruses have an at least 100-fold-decreased ability to reactivate from latency (38). This specific requirement for the v-cyclin in reactivation is even more striking given that the v-cyclin is dispensable for lytic replication, virulence in acute infection, and establishment of latency (38). We have also demonstrated that the v-cyclin is required for persistent infection in gamma interferon (IFN-γ)-

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deficient mice (17), likely as a consequence of its requirement for efficient reactivation from latency (38).

The host immune response plays an essential role in controlling the extent of both latent and persistent infections (26, 27). γHV68 can establish a latent infection in multiple cell types, including B cells, macrophages, and dendritic cells (13, 32, 44). The viral genes involved in this process are not known. Previous analyses of γHV68 have demonstrated a dynamic interplay between viral infection and the host immune response. A striking example of this is revealed by γHV68 infection of B-cell-deficient mice, which lack both B cells as a major latency reservoir (13, 32) and B-cell-dependent immune responses. Analysis of these mice and other antibody-deficient mice has demonstrated that antibody can regulate the levels of reactivation and latency in these mice (16, 20). On the basis of these observations, it has been proposed that in certain antibody-deficient hosts, ongoing reactivation and reseeding are critical for the maintenance of high levels of latently infected cells (16). Notably, different immunodeficiencies can result in a failure to control chronic infection. For example, both CD8 T-cell-deficient ($CD8^{-/-}$) and B-cell-deficient (MuMT) mice fail to control γHV68 latency and reactivation to similar extents (36, 41, 43).

Given the dynamic nature of chronic infection and its regulation by the host, reactivation from latency is likely to play an important role in successful chronic infection. To test this, we have analyzed the role of the v-cyclin in the following parameters of chronic γHV68 infection: reactivation from latency, maintenance of latent infection, persistent infection, and long-term mortality. We have performed these analyses with both CD8 T-cell-deficient ($CD8^{-/-}$) and B-cell-deficient (MuMT) mice. A significant difference between these mice is the absence of B cells, a major latency reservoir, in B-cell-deficient mice. By comparison of these different hosts, these studies further establish that v-cyclin and v-cyclin-dependent reactivation are critical for maintenance of gammaherpesvirus latency and chronic disease in the absence of B cells.

MATERIALS AND METHODS

Viruses and tissue culture. γHV68 clone WUMS (ATCC VR1465) and v-cyclin-deficient viruses were passaged and grown and their titers were determined on NIH 3T12 cells as previously described (39). This study used two γHV68 virus strains containing the wild-type (wt) v-cyclin gene: (i) parental wt γHV68 (strain WUMS) from which the v-cyclin-deficient viruses were generated, and (ii) the v-cyclin marker rescue virus (v-cyclin.MR), in which wt v-cyclin sequences were reintroduced into the v-cyclin.LacZ mutant (38). Wt γHV68 and v-cyclin.MR have been phenotypically identical in all of the assays tested to date (38). The v-cyclin-deficient viruses include (i) the v-cyclin.LacZ mutant, in which the v-cyclin gene was disrupted by insertion of a LacZ expression cassette under control of the cytomegalovirus immediate-early promoter, and (ii) the v-cyclin .Stop mutant, in which the v-cyclin gene was disrupted by insertion of a translation stop and frameshift linker cassette (38).

Mice, infections, and organ harvests. MuMT (B-cell-deficient) mice (21, 41) backcrossed onto a C57BL/6 background and C57BL/6J (BL/6) mice deficient in the CD8 α chain ($CD8^{-/-}$) (15) (Jackson Laboratory catalog no. 002665) were bred and maintained at Washington University School of Medicine, St. Louis, Mo., in accordance with all federal and university policies. BL/6 mice were purchased from The Jackson Laboratory (catalog no. 000664). Unless otherwise stated, mice were age and sex matched and infected at 7 to 10 weeks of age with 10^6 PFU of γHV68 by intraperitoneal injection in 0.5 ml of complete Dulbecco modified Eagle medium (38). Peritoneal cells and splenocytes were harvested and processed as previously described (38) from groups of five mice per experiment.

Frequency of cells harboring the γHV68 genome. The frequency of cells harboring the γHV68 genome was determined by a limiting-dilution, nested-PCR assay that amplifies a γHV68 gene 50 sequence with single-copy sensitivity (43, 44). Briefly, peritoneal cells and splenocytes were frozen in 10% dimethyl sulfoxide at −80°C, thawed, counted, resuspended in isotonic buffer, and serially diluted into 96-well PCR plates. Uninfected NIH 3T12 cells were added so that each well contained a total of 10^4 cells. Cells were then lysed in detergent and digested with proteinase K prior to nested PCR analysis (38). Twelve PCRs were analyzed for each cell dilution, with six dilutions per sample per experiment. Control reaction mixtures in each experiment included uninfected cells alone (six reactions per plate) or cells with 10 copies, 1 copy, or 0.1 copy of plasmid DNA containing the target sequence (six reactions per plate each). There were no false-positive reactions in the assays reported here, and all of the assays demonstrated approximately one-copy sensitivity for plasmid DNA.

Frequency of cells reactivating from latency ex vivo. The frequency of cells reactivating from latency ex vivo was determined with a limiting-dilution reactivation assay (38). Briefly, peritoneal cells and splenocytes were harvested 180 to 365 days postinfection (p.i.) and plated in serial twofold dilutions (starting at 4×10^4 peritoneal cells) onto mouse embryonic fibroblast monolayers in 96-well plates. Twenty-four wells were plated per dilution, and 12 dilutions were plated per sample. Wells were scored microscopically 21 days later for viral cytopathic effect. Preformed virus in tissues was detected by parallel plating of mechanically disrupted cells. Mechanical disruption kills >99% of the cells but does not inactivate the virus (41) and therefore allows quantification of preformed, persistent virus.

Measurement of chronic viral persistence in lungs. Viral persistence in the lung was measured by pooling the lower left lobe of the lung from each animal and mechanically disrupting the tissue as previously described (38). Samples were then diluted to 5 ml in complete medium and plated in twofold dilutions onto mouse embryonic fibroblasts in 96-well plates, with 24 wells plated per dilution and eight dilutions plated per experimental group.

Statistical methods. All data were analyzed with GraphPad Prism software (GraphPad Software, San Diego, Calif.). Frequencies were calculated on the basis of the Poisson distribution by determining the cell number at which 63.2% of the wells scored positive for either reactivating virus or presence of the viral genome. Data were subjected to nonlinear regression analysis with a sigmoidal dose-response equation to obtain the single-cell frequency for each limiting-dilution analysis (9). In this analysis, best-fit values of two data curves were compared with an unpaired *t* test of log 50% effective concentrations ± the standard error of the mean to calculate *P* values, as reported in the figure legends. For values not reported, there were no statistically significant differences. Mice were scored as dead at the time of death or when sacrificed if moribund, and survival data were analyzed by the use of the Mantel-Haenszel test, with death as the primary variable.

RESULTS

The v-cyclin is not required for long-term maintenance of latency following infection of immunocompetent BL/6 mice. We have previously demonstrated that the v-cyclin is required for efficient reactivation from latency but not for the establishment of latency at 42 days p.i. (38). Given the potential role of reactivation in chronic infection (16, 20), we initiated a detailed analysis of long-term infection with viruses sufficient or deficient in v-cyclin function. BL/6 mice were infected and analyzed for (i) the frequency of cells that reactivate the virus ex vivo and (ii) the frequency of cells that harbor the viral genome. At 6 months p.i., two viruses containing wt v-cyclin, wt γHV68 and v-cyclin.MR, reactivated equivalently from latently infected peritoneal cells when the cells were cultured ex vivo (wt reactivation frequency, 1 in 81,900; Fig. 1A). Consistent with previous studies that analyzed latency at earlier times p.i. (18, 38), two v-cyclin-deficient viruses, v-cyclin.LacZ and v-cyclin.Stop, failed to reactivate from infected peritoneal cells (Fig. 1A). Despite the severe defect of v-cyclin-deficient viruses in reactivation from latency, the frequencies of viral genome-containing peritoneal cells were very similar between wt and v-cyclin-deficient viruses (wt γHV68, 1 in 11,200; v-

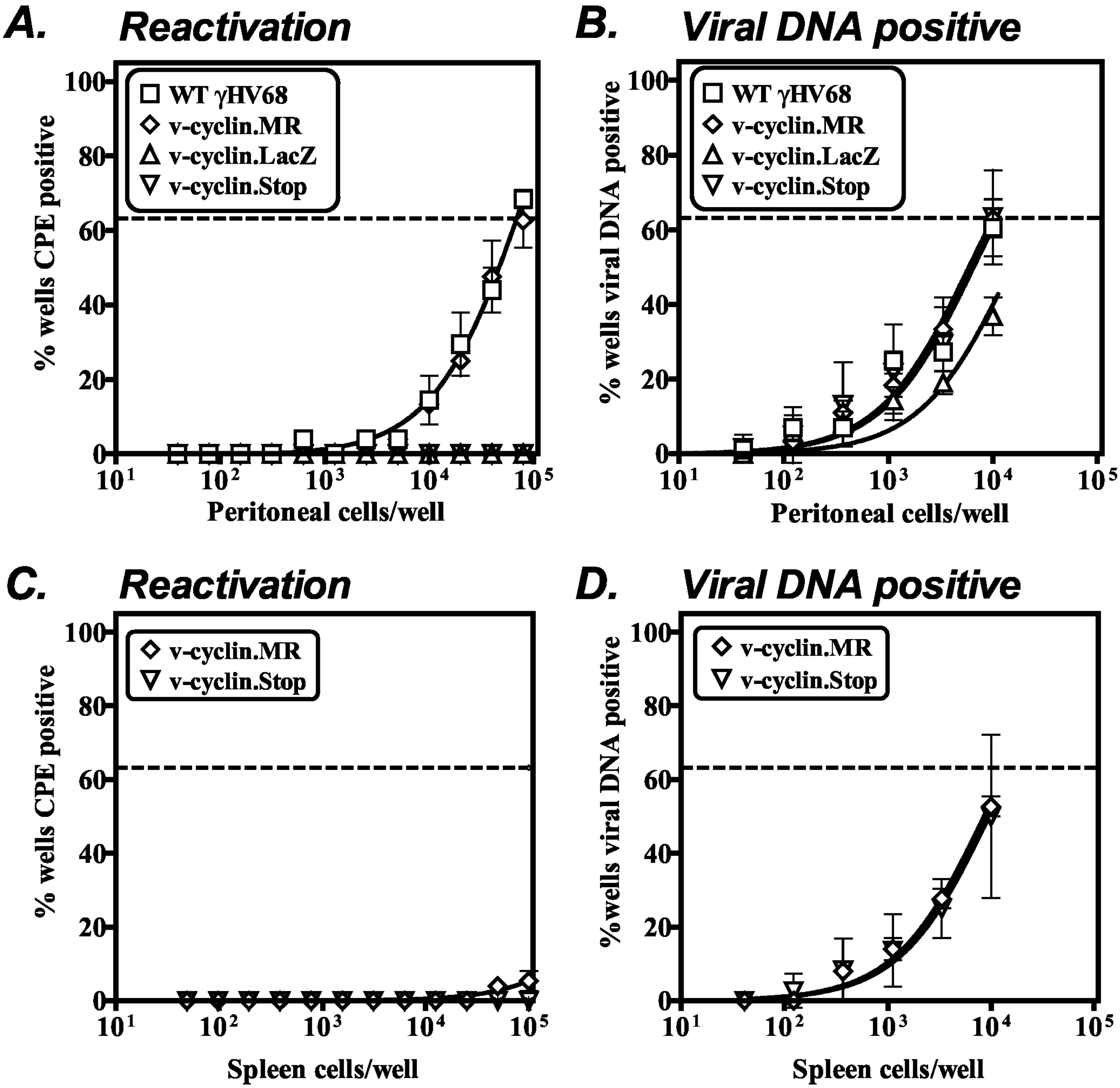

FIG. 1. The v-cyclin is required for reactivation from latency but not for maintenance of latency at 6 months to 1 year p.i. of C57BL/6 mice. BL/6 mice were infected with wt γHV68 (□), v-cyclin.MR (◇), v-cyclin.LacZ (Δ), or v-cyclin.Stop (▽) for quantification of the frequency of cells reactivating virus (A and C) and the frequency of viral genome-positive cells (B and D) in either peritoneal cells (A and B) or splenocytes (C and D). For reactivation analyses, closed symbols denote mechanically disrupted samples (uniformly negative here and coincident with open triangles and the *x* axis). The data shown represent independent experiments, as indicated (wt γHV68, $n = 2$; v-cyclin.MR, $n = 3$; v-cyclin.LacZ, $n = 2$; v-cyclin.Stop, $n = 1$). Curve fit lines were derived by nonlinear-regression analysis, and symbols represent the mean ± the standard error of the mean (error bars) of data. The dashed line at 63% is the value used to calculate the frequency of reactivating cells or viral genome-positive cells by the Poisson distribution. Statistically significant differences: A, $P < 0.0001$ for both v-cyclin.LacZ and v-cyclin.Stop compared to wt γHV68; B, $P < 0.001$ for v-cyclin.LacZ compared to wt γHV68. CPE, cytopathic effect.

cyclin.MR, 1 in 10,100; v-cyclin.Stop, 1 in 10,300; v-cyclin .LacZ, extrapolated to be ca. 1 in 25,800; Fig. 1B). Similar results were obtained with splenocytes harvested from these mice (Fig. 1C and D), although, as previously shown, little reactivation was observed in splenocytes infected with either wt γHV68 or v-cyclin-deficient viruses at earlier times p.i. (38). Thus, although the lack of v-cyclin function leads to a severe impairment in virus reactivation from latency, this did not have a significant impact on the overall maintenance of latently infected cells in either the peritoneum or spleen at 6 months p.i. On the basis of these data, in immunocompetent BL/6 mice, the v-cyclin, and potentially v-cyclin-dependent reactiva-

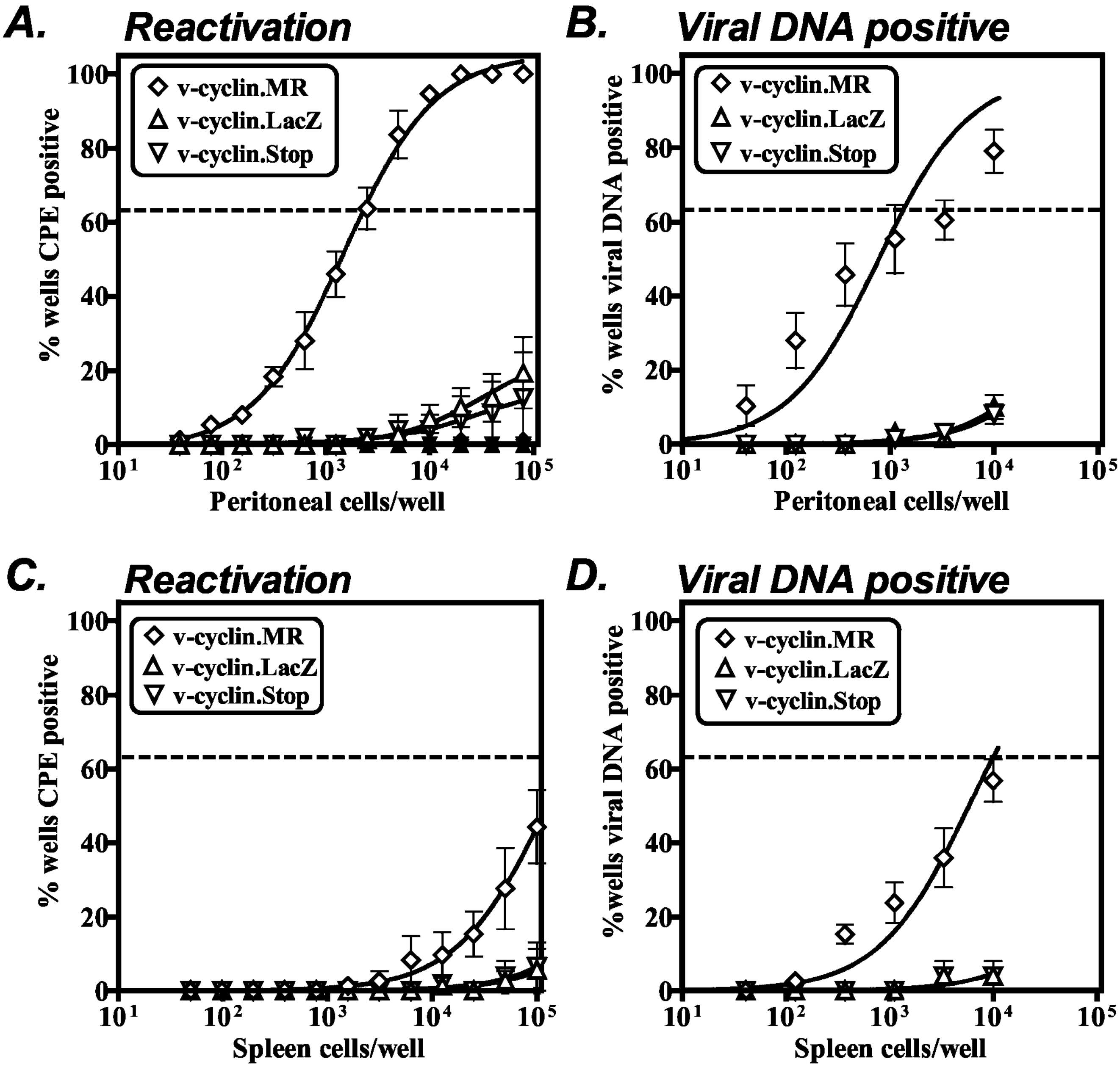

FIG. 2. The v-cyclin is required for both reactivation from latency and maintenance of latency at 6 months to 1 year p.i. in B-cell-deficient mice. MuMT mice were infected with v-cyclin.MR (◇), v-cyclin.LacZ (Δ), or v-cyclin.Stop (▽) virus for quantification of the frequency of cells reactivating virus (A and C) and the frequency of viral genome-positive cells (B and D) in either peritoneal cells (A and B) or splenocytes (C and D). For reactivation analyses, closed symbols denote mechanically disrupted samples. Data represent independent experiments, as indicated (v-cyclin.MR, $n = 3$; v-cyclin.LacZ, $n = 3$; v-cyclin.Stop, $n = 2$). Data are presented and analyzed as described in the legend to Fig. 1. Statistically significant differences: $P < 0.0001$ for both v-cyclin.LacZ and v-cyclin.Stop compared to v-cyclin.MR in A, B, C, and D. CPE, cytopathic effect.

tion, is not essential for long-term (6 months) maintenance of γHV68 latency.

The v-cyclin is critical for long-term maintenance of latency in B-cell-deficient mice. The outcome of gammaherpesvirus infection is heavily influenced by the immune status of the host, and therefore the dynamics and regulation of chronic infection may vary significantly between immunocompetent and immunocompromised hosts. Chronic infection of immunocompromised MuMT mice differs from that of normal immunocompetent mice, since MuMT mice fail to efficiently control latent infection and succumb to long-term infection (43). MuMT mice also lack B cells, which serve as a major latency reservoir for γHV68 (13, 32). γHV68 has the capacity to establish a latent infection in other cell types, including macrophages, dendritic cells, and, potentially, lung epithelium cells (13, 30, 44).

We therefore analyzed latency and reactivation in MuMT mice. By 6 months to 1 year p.i., wt γHV68 was capable of

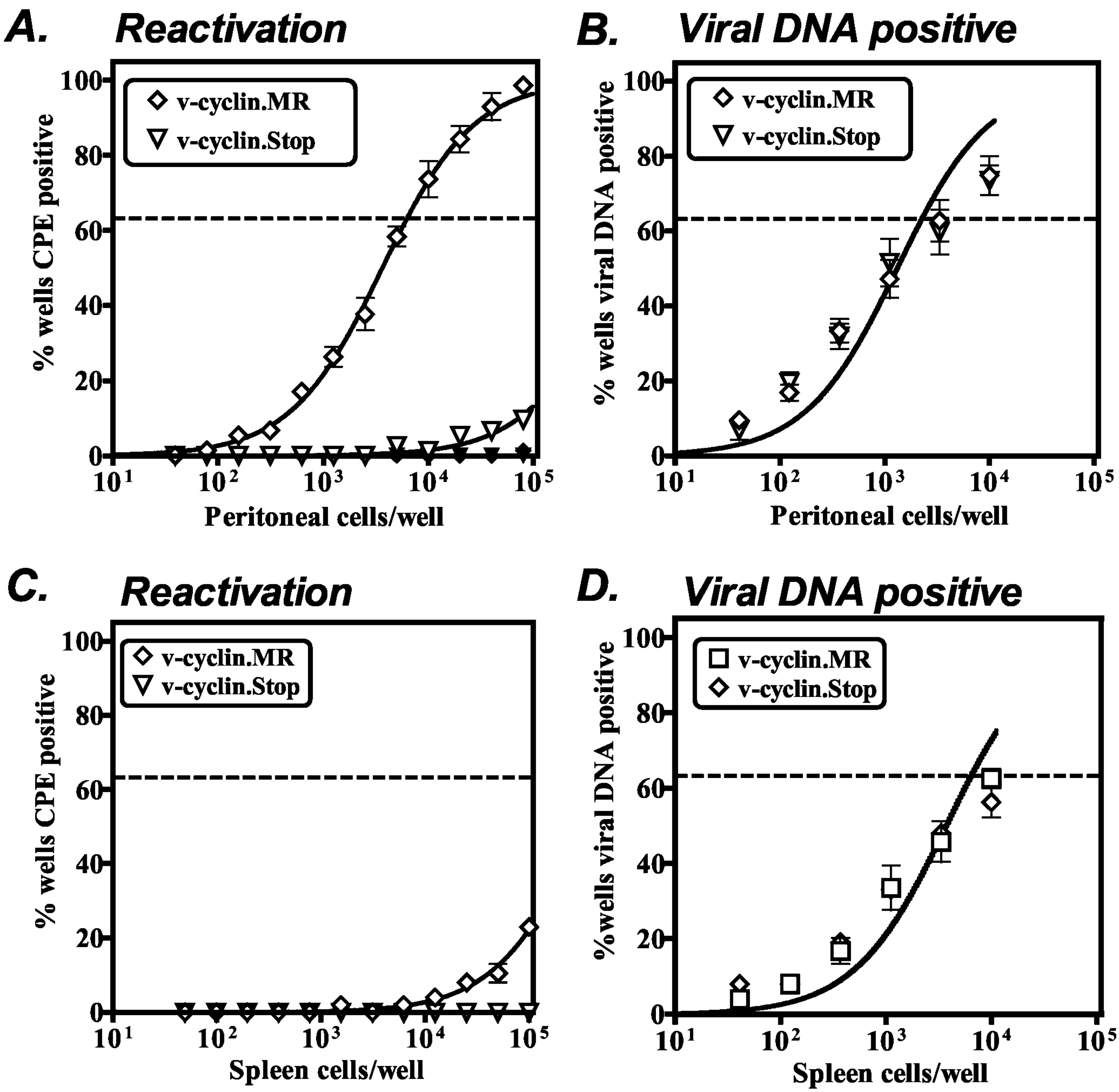

FIG. 3. The v-cyclin is required for reactivation from latency but not for maintenance of latency at 6 months p.i. in CD8-deficient mice. $CD8^{-/-}$ mice were infected with v-cyclin.MR (◇) or v-cyclin.Stop (▽) virus for quantification of the frequency of cells reactivating virus (A and C) and the frequency of viral genome-positive cells (B and D) in either peritoneal cells (A and B) or splenocytes (C and D). For reactivation analyses, closed symbols denote mechanically disrupted samples. The data shown represent independent experiments, as indicated (v-cyclin.MR, $n = 3$; v-cyclin.Stop, $n = 3$). Data are presented and analyzed as described in the legend to Fig. 1. Statistically significant differences: A, $P < 0.0001$ for v-cyclin.Stop compared to v-cyclin.MR. CPE, cytopathic effect.

reactivating from peritoneal cells (1 in 2,300 cells; Fig. 2A) whereas v-cyclin-deficient viruses demonstrated a severe defect in reactivation from peritoneal cells (<1 in 100,000, Fig. 2A). Strikingly, however, analysis of the frequency of latently infected cells in MuMT mice demonstrated that v-cyclin-deficient viruses also have a significant defect in the maintenance of latently infected cells. This defect resulted in a greater-than-100-fold decrease in the frequency of v-cyclin-deficient latently infected cells by 6 months p.i. compared to that of latently wt γHV68-infected cells (wt γHV68, 1 in 1,300; v-cyclin-deficient viruses, <1 in 100,000; Fig. 2B). Splenocytes harvested from these mice revealed a similar defect of v-cyclin-deficient viruses both in reactivation from latency and in maintenance of latently infected cells (Fig. 2C and D). This is the first example of a herpesvirus mutant that can establish a latent infection in all of the mice tested to date (Fig. 1 and 3) (38) but is unable

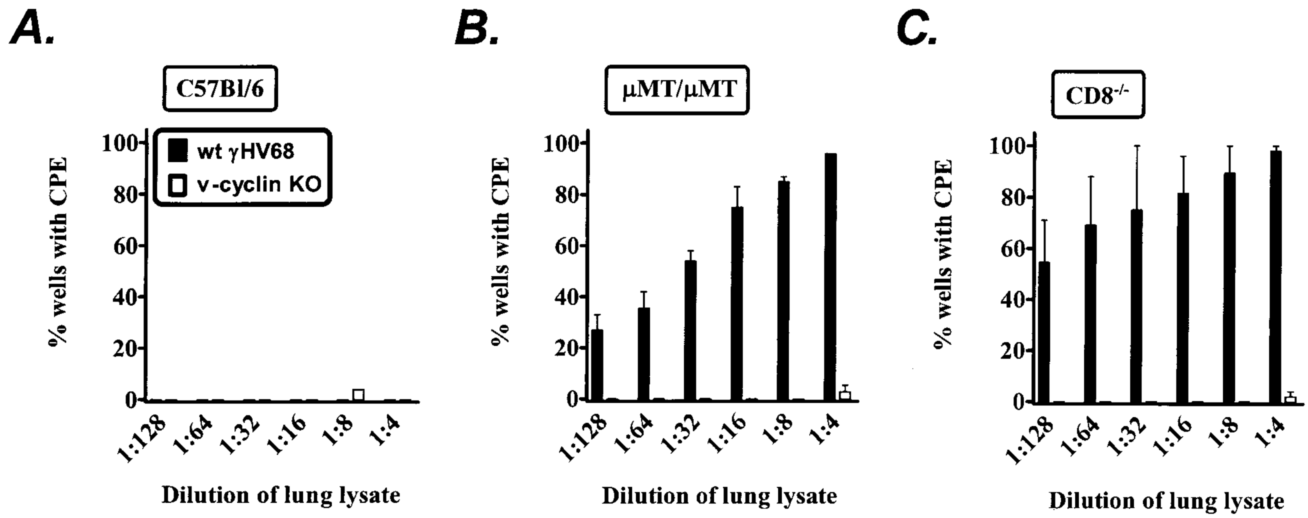

FIG. 4. The v-cyclin is required for persistent viral replication in the lungs of immunocompromised mice. BL/6 (A), MuMT (B), and $CD8^{-/-}$ (C) mice were infected with either wt γHV68 (black-filled bars) or v-cyclin-deficient γHV68 (open bars). The data shown represent independent experiments, as indicated (BL/6 mice, wt γHV68, $n = 2$; BL/6 mice, v-cyclin deficient, $n = 1$; MuMT mice, wt γHV68, $n = 3$; MuMT mice, v-cyclin deficient, $n = 3$; $CD8^{-/-}$ mice, wt γHV68, $n = 2$; $CD8^{-/-}$ mice, v-cyclin deficient, $n = 2$). CPE, cytopathic effect; KO, knockout.

to efficiently maintain a latent infection (Fig. 2) in B-cell-deficient mice. The failure to maintain latency in MuMT mice infected with v-cyclin-deficient virus could be due to the lack of B cells or to a general feature of immunocompromised hosts, that is, the failure to control latent infection.

The v-cyclin is not required for long-term maintenance of latency following infection of immunocompromised CD8-deficient mice. To test whether failure of immunocompromised mice to control latent infection results in a common failure to maintain latency, we analyzed γHV68 latency and reactivation in $CD8^{-/-}$ mice. Previous analyses of $CD8^{-/-}$ and MuMT mice infected with wtγHV68 have shown that both $CD8^{-/-}$ and MuMT mice fail to appropriately control γHV68 latency and reactivation, resulting in similar increases in the frequency of latently infected cells and hyperreactivation from latency (36, 43). Previously, we have found that, at 42 days p.i., the v-cyclin is required for efficient reactivation but not for the establishment of latently infected cells in both $CD8^{-/-}$ and MuMT mice (data not shown and reference 38).

We therefore analyzed latency 6 months p.i. in $CD8^{-/-}$ mice. We observed detectable reactivation from peritoneal cells in mice infected with wt γHV68 (1 in 6,200 cells) but very little reactivation from peritoneal cells of mice infected with v-cyclin-deficient virus (<1 in 100,000) (Fig. 3A). Similar to the results obtained with BL/6 mice, the frequency of latently infected cells was indistinguishable between mice infected with wt γHV68 (1 in 2,300) and those infected with v-cyclin-deficient virus (1 in 2,200) (Fig. 3B). Similar results were observed with splenocytes harvested from these mice (Fig. 3C and D). These data demonstrate that v-cyclin-deficient viruses are efficiently maintained in both immunocompetent (Fig. 1B) and immunocompromised (Fig. 3B) hosts and that the v-cyclin is not a general requirement for long-term maintenance of γHV68 infection.

On the basis of these data, the v-cyclin is critical for reactivation from latency in both immunocompetent BL/6 mice and immunocompromised MuMT and $CD8^{-/-}$ mice at 6 months p.i. (Fig. 1, 2, and 3). Furthermore, there is a striking failure in long-term maintenance of latency of v-cyclin-deficient viruses in B-cell-deficient mice but not in BL/6 or $CD8^{-/-}$ mice. These observations are consistent with the hypothesis that γHV68 reactivation and reseeding of latency reservoirs are required for maintenance of chronic infection in the absence of B cells. In contrast, v-cyclin-deficient viruses are efficiently maintained in the presence of B cells. Since the v-cyclin is dispensable for maintenance of latency in BL/6 and $CD8^{-/-}$ mice, this further suggests that either (i) B cells are a long-lived latency reservoir that does not require continual reseeding to be maintained or (ii) reactivation of γHV68 from B cells does not require v-cyclin function.

The v-cyclin is required for ongoing virus replication in the lungs of immunocompromised mice. Another important parameter of chronic gammaherpesvirus infection is the establishment of a persistent infection at certain sites in the host. We have previously demonstrated that γHV68 mutants impaired in reactivation from latency (v-cyclin- or v-bcl-2-deficient viruses) are also defective in persistent virus replication in the peritoneum of IFN-$\gamma^{-/-}$ mice (17). In addition, persistent virus replication in these mice correlated with increased mortality and viruses lacking v-cyclin or v-bcl-2 expression showed improved long-term survival (17). On the basis of these observations, there appears to be an intimate link between reactivation and persistent infection.

To further understand the requirements for v-cyclin in persistent infection, we quantified persistent infection by using mechanically disrupted cells from both the peritoneum and lungs of infected BL/6, $CD8^{-/-}$, and MuMT mice. Notably, we detected no persistent production of virus in the peritoneum of BL/6, $CD8^{-/-}$, or MuMT mice at 6 months p.i. (see disrupted samples, as indicated by closed symbols in Fig. 1A, 2A, and 3A), despite ready detection of cells carrying viral DNA (Fig. 1B, 2B, and 3B). Latently infected cells carry episomal viral

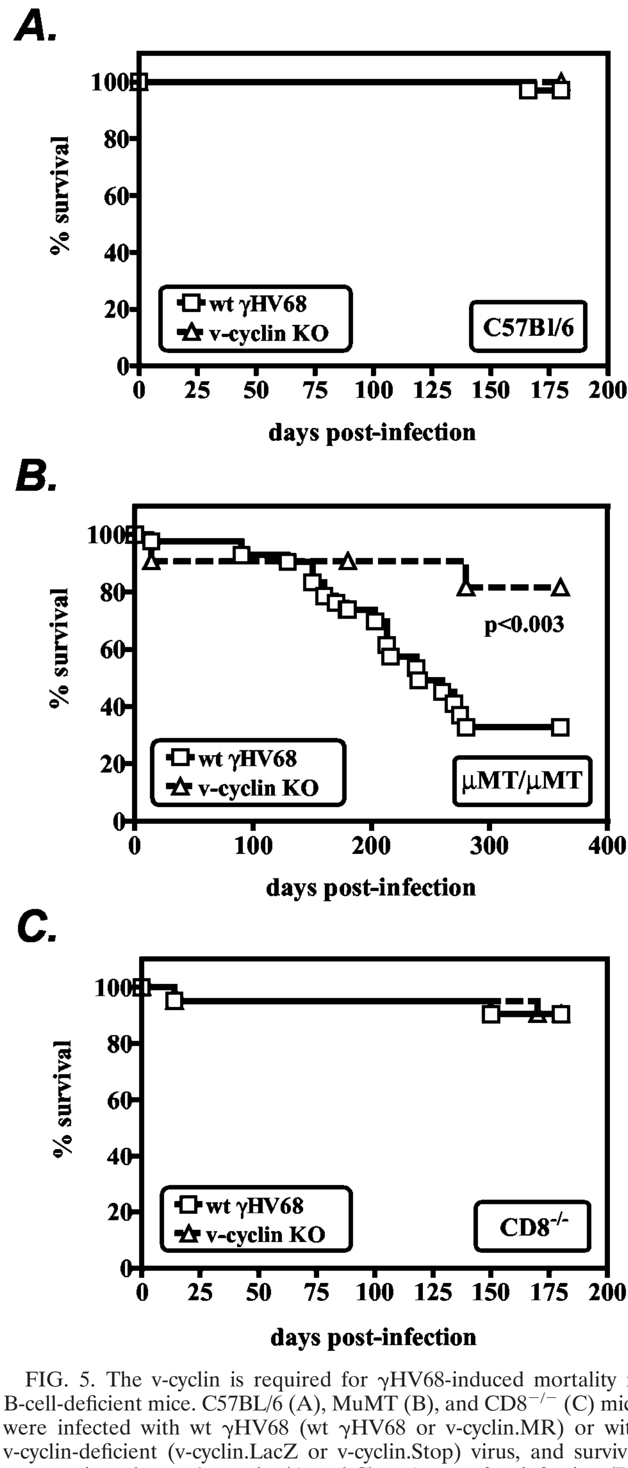

FIG. 5. The v-cyclin is required for γHV68-induced mortality in B-cell-deficient mice. C57BL/6 (A), MuMT (B), and CD8$^{-/-}$ (C) mice were infected with wt γHV68 (wt γHV68 or v-cyclin.MR) or with v-cyclin-deficient (v-cyclin.LacZ or v-cyclin.Stop) virus, and survival was monitored over 6 months (A and C) or 1 year after infection (B). The increased survival of B-cell-deficient mice infected with v-cyclin-deficient virus versus that of mice infected with wt γHV68 or v-cyclin.MR virus was statistically significant ($P < 0.008$ at 6 months p.i.; $P < 0.003$ at 1 year p.i.). The data shown were compiled from infection of mice as follows: BL/6, wt γHV68, $n = 34$; BL/6, v-cyclin deficient, $n = 27$; MuMT, wt γHV68, $n = 42$; MuMT, v-cyclin deficient, $n = 43$; CD8$^{-/-}$, wt γHV68, $n = 20$; CD8$^{-/-}$, v-cyclin deficient, $n = 20$. KO, knockout.

DNA (30) but fail to replicate viral DNA or produce infectious virus. We also examined the lungs for viral persistence on the basis of previous reports of persistent virus replication in the lungs of γHV68-infected MuMT mice (30) and the association of γHV68 with pneumonia in immunocompromised mice (7). Consistent with the tight control of chronic γHV68 infection in BL/6 mice, there was no evidence of virus replication in the lungs of BL/6 mice at 6 months p.i. with either wt γHV68 or v-cyclin-deficient γHV68 (Fig. 4A). In contrast, infection of both MuMT and CD8$^{-/-}$ mice with wt γHV68 resulted in significant levels of persistent virus replication at 6 months p.i. (Fig. 4B and C). Notably, this persistent infection of the lungs required functional v-cyclin since there was little or no detectable persistent virus replication in the lungs of MuMT or CD8$^{-/-}$ mice infected with v-cyclin-deficient viruses (Fig. 4B and C). This failure of v-cyclin-deficient viruses to persist in the lungs likely results from the role of the v-cyclin in reactivation from latency rather than a defect in ongoing lytic replication, since (i) the v-cyclin has been shown to be critical for reactivation from latency (18, 38) and (ii) there are no discernible defects in acute virus replication in the lungs, livers, or spleens of mice infected with v-cyclin-deficient viruses (38).

The v-cyclin is required for γHV68-induced mortality in B-cell-deficient mice. We have previously observed that 50% of MuMT mice succumb to chronic γHV68 infection by 1 year p.i. (43), providing a model for chronic gammaherpesvirus-induced mortality in an immunocompromised host. To test whether v-cyclin-deficient viruses are impaired in the ability to induce chronic mortality, we compared the survival of BL/6, CD8$^{-/-}$, or MuMT mice infected with either wt or v-cyclin-deficient γHV68. Infection of BL/6 mice with either wt γHV68 or v-cyclin-deficient virus resulted in virtually no mortality (Fig. 5A). Interestingly, although CD8$^{-/-}$ mice are immunocompromised and fail to effectively control both reactivation from latency and persistent lung infection, neither wt γHV68-infected nor v-cyclin-deficient virus-infected CD8$^{-/-}$ mice exhibited significant mortality over 6 months of infection (Fig. 5C). In contrast, more than 60% of MuMT mice infected with wt γHV68 succumbed to chronic infection (Fig. 5B). Remarkably, the mortality of MuMT mice infected with v-cyclin-deficient virus was significantly decreased, with less than 20% of the animals dying by 1 year p.i. (Fig. 5B; $P < 0.008$ at 6 months p.i., $P < 0.003$ at 1 year p.i.). The fact that mortality is decreased rather than eliminated is consistent with a significant, but not essential, role for the v-cyclin in reactivation from latency, as previously reported (38, 17). On the basis of these data, the v-cyclin is critical for lethality in a model of gammaherpesvirus-induced mortality in immunocompromised animals. These data further suggest that ongoing reactivation from latency is critical for lethality in the MuMT model of chronic infection and that ongoing reactivation and its resolution are inherently different in CD8$^{-/-}$ mice.

DISCUSSION

Chronic gammaherpesvirus infection exists in a delicate balance between host and viral factors. One of the most important mechanisms of gammaherpesvirus regulation is the host immune system. While multiple cell types and effector mechanisms are responsible for controlling gammaherpesvirus infection (5, 11, 40), there is an emerging appreciation that different mechanisms of immunity regulate different stages and sites of chronic infection (e.g., see reference 36). This is nicely illustrated by the common inability of CD8$^{-/-}$ and MuMT mice to control latency, reactivation, and persistence, yet only MuMT mice display significant virus-associated mortality. While the basis of this differential mortality is unknown, it is clear that viral persistence is not strictly correlated with mortality, as even v-cyclin-infected animals with virtually no detectable persistence succumb to infection. One possibility is that there is viral persistence at an undetected site or at an extremely low level that predisposes to mortality. Interestingly, MuMT mice are also susceptible to large-vessel arteritis (42) in chronic infection with wt γHV68 but with a decreased incidence following infection with v-cyclin-deficient viruses (17). Induction of arteritis in CD8$^{-/-}$ mice has not been described to date. Chronic infection may also result in immune-mediated pathology involving CD8 T cells, as has been shown in γHV68-infected spleens of IFN-γ receptor$^{-/-}$ mice at early times (12). Alternatively, antibody may be important for quenching of reactivation events and for prevention of systemic spread and infection of distant tissues in vivo. In contrast, CD8 T cells may primarily provide local control of virus reactivation by killing infected cells. Consistent with this hypothesis, recent reports have demonstrated that passive transfer of immune antisera restores an increased level of immunological control on reactivation from latency and persistence in mice lacking protective antibodies (16, 20).

Notably, the dynamics of chronic infection appear to be highly regulated by the host immune response. While immune status regulates reactivation and persistence, it may also contribute to the establishment of qualitatively different forms of latency, as has been demonstrated by the distinct transcriptional programs of EBV infection in humans (25). Failure to control chronic infection is exemplified in MuMT mice, which exhibit hyperreactivation from latency and an apparent ongoing reseeding and replenishment of the latency pool derived from spontaneous reactivation in vivo. This model is supported by three pieces of data: (i) v-cyclin-deficient viruses that are defective in reactivation fail to be maintained efficiently in MuMT mice, (ii) passive transfer of neutralizing antibodies to virion proteins results in decreased reactivation and latency in MuMT mice (16, 20), and (iii) treatment of infected MuMT mice with an antiviral inhibitor of lytic replication results in a decreased frequency of latently infected cells (16).

These observations suggest a model in which B cells have a critical role as a long-lived reservoir of γHV68 latency exempt from the requirements of v-cyclin-mediated reactivation and reseeding and that these cells are unique relative to other latently infected cell types, including macrophages, dendritic cells, and lung epithelial cells. Previous work by our group has established that macrophages are a major latency reservoir in the peritoneum of both normal BL/6 mice and B-cell-deficient mice (44). The requirement for the v-cyclin in B-cell-deficient mice suggests that γHV68 genes may have cell type-specific roles and that the B-cell latency reservoir may be independent of v-cyclin and/or v-cyclin-dependent reactivation. In support of this concept, recent work has demonstrated that γHV68 latency is found predominantly in germinal-center B cells (14) and memory B cells (D. Willer and S. H. Speck, unpublished data) (14). Memory B cells represent a long-lived cell type that may allow long-term maintenance of latency independent of reactivation. This is consistent with EBV, a human gammaherpesvirus in which long-term latency reservoirs have also been identified in memory B cells (34). The requirement for the v-cyclin in long-term maintenance of latency in the absence of B cells is consistent with either a gradual accumulation of latency exclusively within B cells in normal mice infected with v-cyclin-deficient virus or with ongoing latent infection of multiple cell types with reseeding from the B-cell compartment. While the v-cyclin and v-cyclin-dependent reactivation are not required for long-term latency in the presence of B cells, the v-cyclin is, in fact, expressed in these latently infected B cells (14) and may have a function that has yet to be described.

Vaccination against gammaherpesviruses has been a major goal in preventing gammaherpesvirus-associated morbidity and mortality (22, 28, 29); however, it has not yet proven efficient in preventing chronic disease (4). Recent studies have demonstrated effective protection from wt γHV68 latency (35) by vaccination with a v-cyclin-deficient live attenuated virus; this promising strategy has been predicted to result in prevention of chronic disease. However, it is critical to continue to consider alternative targets and approaches to control established chronic gammaherpesvirus infection. This study establishes that the v-cyclin and v-cyclin-dependent reactivation are critical for chronic gammaherpesvirus-associated disease in MuMT mice. Together with the previous demonstration that the v-cyclin and v-cyclin-dependent reactivation are critical to chronic gammaherpesvirus-associated disease in IFN-γ$^{-/-}$ mice, this work suggests that the v-cyclin and v-cyclin-dependent reactivation are potential points of intervention in chronic gammaherpesvirus disease. These data are consistent with chronic gammaherpesvirus diseases associated with ongoing persistent infection or reactivation that have been effectively treated with antiviral drugs known to inhibit viral replication (1, 10, 23). The gammaherpesvirus v-cyclins have unique biochemical features (19, 33) distinct from host cyclins that may allow the development of highly specific antiviral therapies for chronic gammaherpesvirus disease associated with reactivation and persistent infection.

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